⑦ * High Performance liquid chromatography :-

* Principle :-

In HPLC, eluent from the solvent reservoir is filtered, pressurised and pumped through the column. A mixture of solutes injected at the top of the column is separated into components. Individual solutes are monitored by the detector and recorded automatically.

* Theory :-

All forms of liquid chromatography (LC) are differential migration processes. where sample components are selectively retained by a stationary phase. LC covers a variety of separation techniques, such as liquid - solid, liquid - liquid, ion - exchange and exclusion chromatography, all involving a mobile liquid phase. liquid-solid chromatography (LSC) is often termed as adsorption chromatography. liquid-liquid (partition) chromatography (LLC) is similar in principle to solvent extraction. LLC is divided into two categories, based on relative polarities of stationary and mobile phases.

i.e, reverse phase chromatography and ion-pair chromatography. In addition to LSC and LLC, liquid chromatography also comprises bonded phase chromatography and gel-permeation, steric exclusion, ion exchange and affinity chromatography. classical liquid column chromatography is characterised by the use of wide-diameter glass columns percolating through the column under gravity. Although several remarkable separations have been achieved, these are generally slow and examination of the recovered fractions can be tedious. However, with the discovery of high pressure pumps operating at pressures upto 2.07×10^7 Nm^{-2} (3000 psi) as well as the knowledge of effluent analysis with refractive index or uv detectors. the technique of high pressure liquid chromatography or high speed liquid chromatography later termed high performance liquid chromatography (HPLC) attained greater significance in separation science. The technique of HPLC was developed by

Csaba Horvath (1964), Kirkland and Huber in 1969. The first mixture to be separated by Horvath group were nucleic acid components associated with thyroid function.

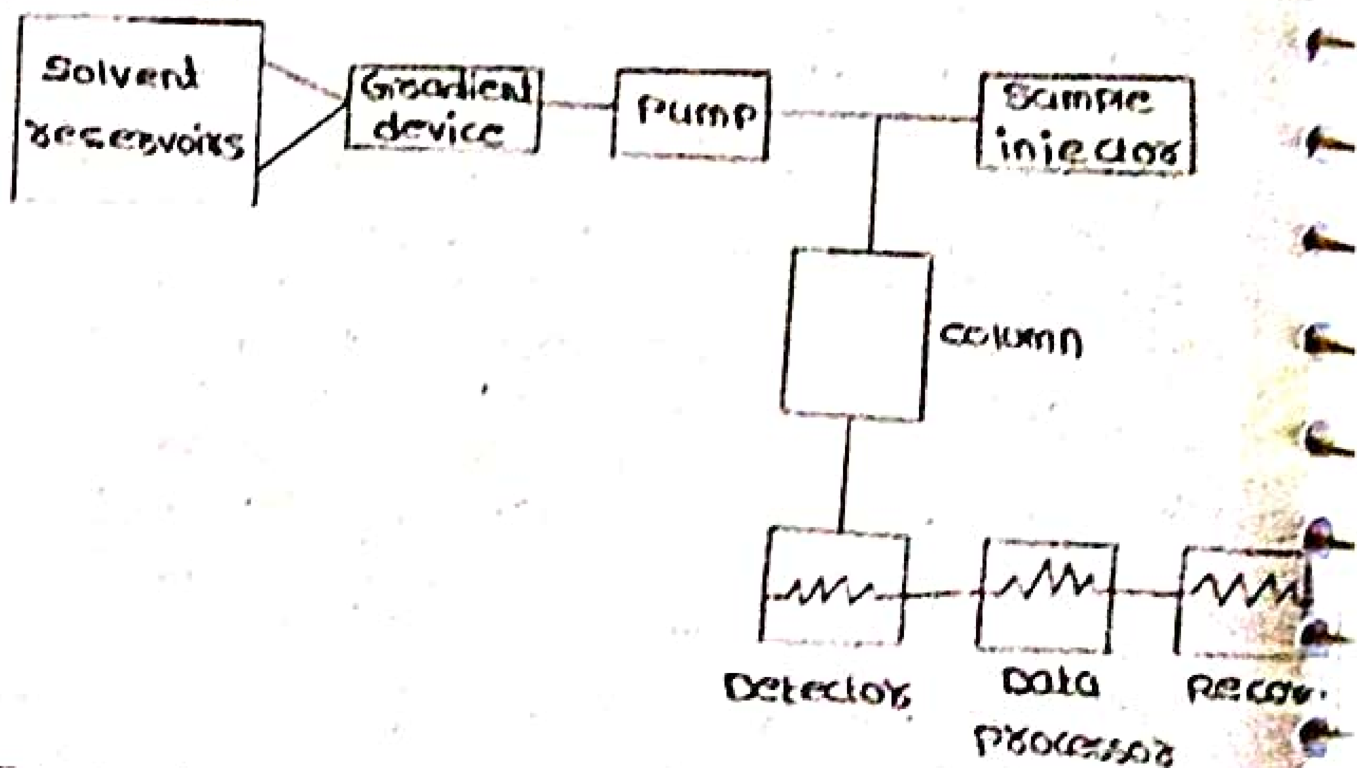
* Instrument description of the different parts of the equipment :-

A typical modern liquid chromatograph consists of following components :

① Solvent delivery system which includes a pump, pressure controls, flow controls and inlet filter.

② Sample injection system.

③ column ④ Detector ⑤ Recorder ⑥ Data control and display.



Block diagram of a modern liquid chromatograph.

separation by HPLC which employs a single solvent is called an isocratic elution. In case two or more solvent systems of significant differences in polarity are used, the method is known as gradient elution. Accordingly the techniques are called isocratic or gradient techniques. modern HPLC unit has provision for more than two solvent reservoirs to release solvents into the mixing chamber at varying rates.

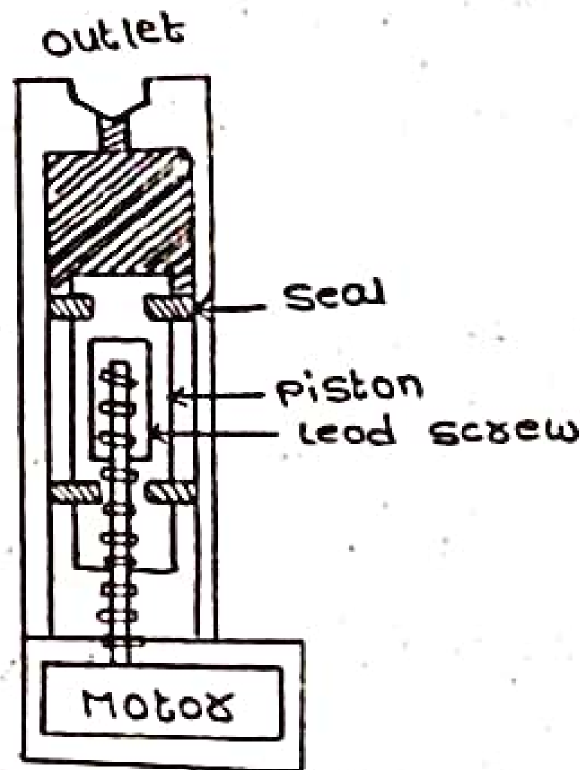
① Solvent Delivery system :-

* High pressure pumps :-

The pump is one of the most important components of HPLC. since it's performance directly affects retention time, reproducibility and detector sensitivity. The pumps deliver a steady stream of solvent from the reservoir to the detector through the column.

The pumps can deliver solvent at a pressure upto 10,000 psi with a flow rate over 50 cm^3 per minute. most of the separations

done by HPLC require pressures between 400 and 1500 psi.



Syring pump

Ⓐ Type of pumps :-

(i) Gas Displacement pump :- (constant pressure pumps). These pumps offer non pulsating flow but have limited solvent capacity.

(ii) Pneumatic pump :- Pneumatic pump contains a mobile phase which is contained in a collapsible container and placed in a vessel. These pumps are inexpensive and pulse free but depend on solvent viscosity and column back pressure.

(iii) syringe pump :- (constant - volume type). These pumps work on the principle of solvent displacement

by a piston mechanically driven at a constant rate in piston chamber of 500 cm^3 capacity. These pumps generate pulseless flow with high pressure (200-475 atm).

③ **mobile phase:-**

The choice of a suitable mobile phase is vital in HPLC. The eluting power of the mobile phase is determined by its overall polarity, the polarity of stationary phase and nature of the sample components. For normal-phase separations eluting power increases with increasing polarity of the solvent while for reverse-phase separations eluting power decreases with increasing solvent polarity.

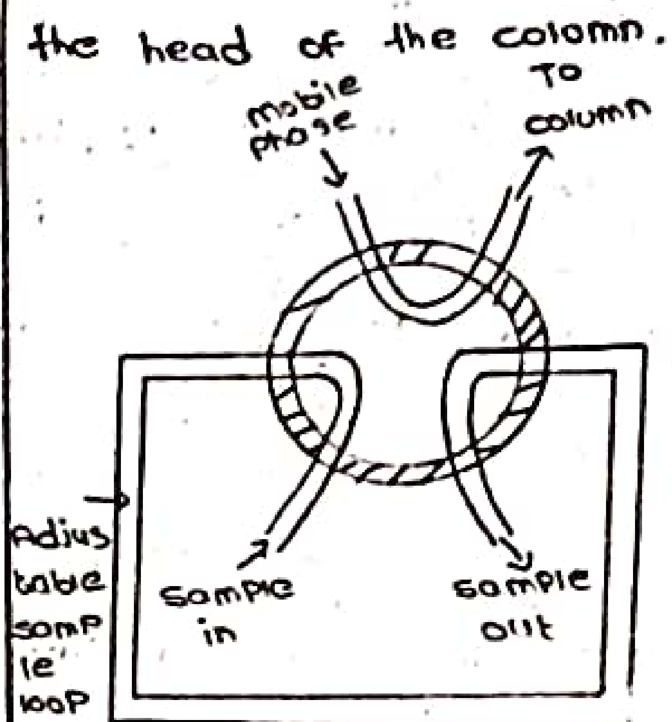
② **sample injection system:-**

The sample (0.002-0.5 cm^3) is introduced into the flowing stream of solvent with an injector by following injection methods.

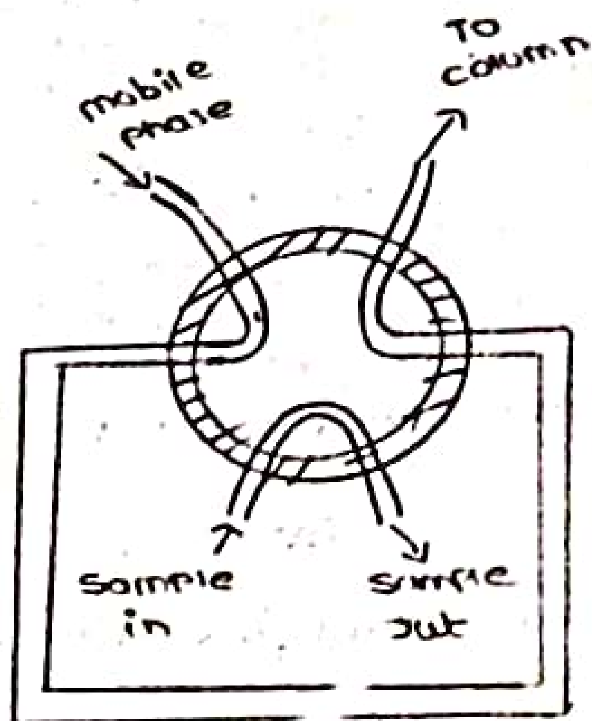
(i) **syringe injection:-** septum injectors allow sample introduction by a high pressure syringe through a self-sealing elastomer septum. However, the problem associated with septum injectors is the

septum, which may yield ghost peaks. This problem can be eliminated by using stop-flow septumless injection.

(ii) **Stop flow injections :-** The flow of solvent is momentarily stopped and the sample is directly injected on the head of the column.



(a) sample mode



(b) injection mode

* **Columns :-**

The columns are made from precision bore polished stainless steel tubing, typical dimensions being 10 to 30 cm long and 4 to 6 mm in internal diameter. Temperature of the column affects speed of affinity, viscosity of the solvent, diffusion and solubility of the sample. Columns are thus used in thermostatic ovens which employ either jacket method or air-circulating

method. The stationary phase or packing is retained at each end by thin stainless steel first with a mesh of ~ 2 μm.

* Detectors :-

In HPLC, the function of detector is to monitor the mobile phase as it emerges from the column. Unlike gas chromatography, HPLC has no reliable detection system. However, suitable detectors can be divided into two categories

(a) Bulk Property Detector :-

These detectors measure the difference in some physical property of the solute in the mobile phase compared to the mobile phase alone e.g, refractive index and conductivity detectors. The latter detector is a universal detector for ionic species and is widely used in ion chromatography and HPLC but they tend to have poor sensitivity and limited range. Such detectors are usually affected by even small changes in the mobile phase composition which precludes the use of gradient elution technique.

(11)

(b) Solute Property detectors :- The detectors are like spectrophotometric, electrochemical and fluorescence detectors. These respond to a particular physical or chemical property of the solute, being independent of the mobile phase. They generally provide high sensitivity (1 in 10^9 being attainable with UV and fluorescence detectors) and a wide linear response range. But on account of their selective natures, more than one detector may be required to meet the requirements. Some commercially available detectors have a number of different detection modes built into a single unit e.g., the perkin - Elmer 3D system combines UV absorption, Fluorescence and conductimetric detection.

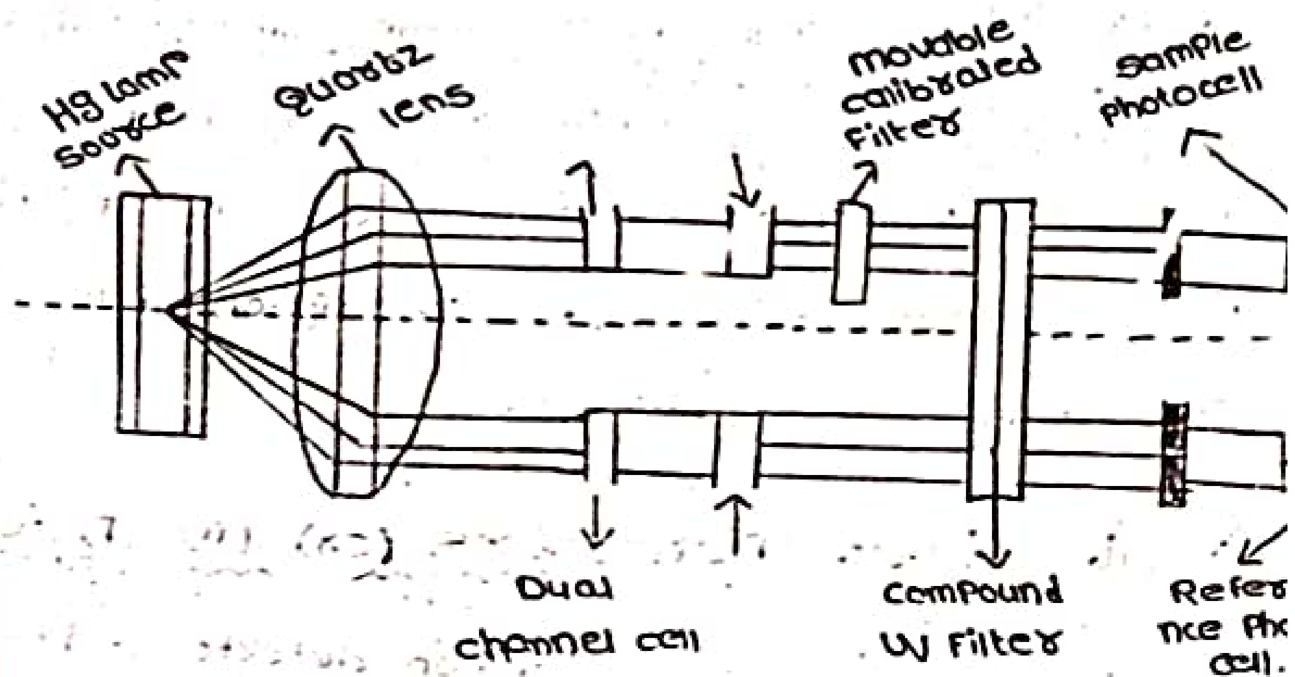
* Ultraviolet Detectors (or) UV Detector:

The UV absorption detectors have been used widely in HPLC. It is based on the principle of absorption of UV visible light as the effluent from the column is passed through a flow cell held in radiation beam. This detector is characterised by

* High sensitivity (detection limit is 1×10^{-9} g cm^{-3} for highly absorbing compounds).

* It is a solute property detector, hence is not sensitive to changes of temperature and flow rate.

* it is suitable for gradient elution work since many solvents used in HPLC do not absorb to any significant extent at the wavelength used for monitoring the column effluent.



The presence of air bubbles in the mobile phase which impair the detector signal, causing spikes on the chromatogram can be eliminated by degassing the mobile phase by ultrasonic vibration. Beside single or dual - wavelength instruments (254 and 280 nm

now variable wavelength detectors (210 - 800 nm)

(12) are used for selective detection.

* Refractometric Detector (or) Refractive Index (RI) Detectors :-

These bulk property detectors are based on the change of refractive index of the eluant from the column with respect to pure mobile phase. Although RI detectors are universal, yet they suffer from some disadvantages such as

(a) lack of high sensitivity.

(b) lack of suitability for gradient elution.

(c) Need strict temperature control (± 0.001 K) to operate at highest sensitivity.

* Fluorescence Detector (or) Luminescence Detectors :-

Fluorescence detectors are sensitive and selective for materials which naturally fluoresce or materials which can be made fluorescent by post - column derivatisation. Since the emission intensity in this technique is directly proportional to excitation intensity, a number

of systems are now using laser sources rather than the xenon lamps, these systems are called laser-induced fluorescence (LIF) detectors. one system uses a frequency-doubled argon ion laser operated at 257 nm. The extremely high intensity, selectivity, low detection limits and spatial resolution of lasers mean that they are well suited to microbore or capillary systems. Detection limit for nucleotides lie between 3×10^{-18} and 10×10^{-18} mol.

* limitations of luminescence detectors :-

(a) compound must be fluorescent.

(b) Detector signal will be swamped by any fluorescent impurities in the sample or solvent.

* Diode Array detector (or) Photodiode arrays (multichannel) detector and charge transfer systems :-

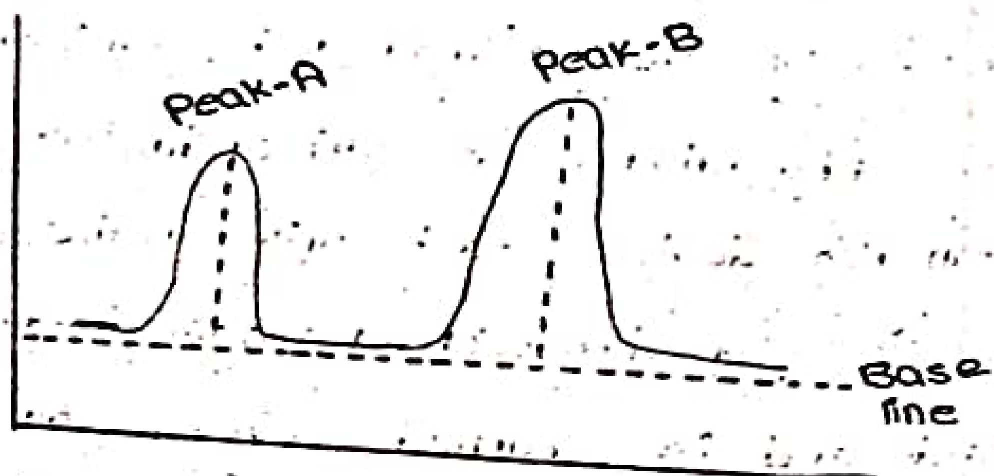
Photodiode arrays (multichannel) detector and charge transfer systems can

record whole of the spectrum many times a second. In these detectors, polychromatic light is passed through the HPLC flow cell and the emerging radiation is diffracted by a grating to fall on the array. This may consist of a series of over a thousand semiconductor sensors, photodiodes or semiconductor charge transfer devices. Each unit of the detector receives a different narrow wavelength band and is scanned several times per second by a microprocessor. The resulting spectrum from the collected signals can be presented on a visual display unit and the changes in the strength and nature of the spectrum are recorded as separate compounds pass through the detector cell.

* Recorder :-

The signals from the detectors are recorded as deviation from a base line. Two open recorders are used with instruments having two detectors. The peak position along the curve, relative to the starting point,

denotes the Particular component. With proper calibration, the height or area of peak is a measure of the amount of the component present in the sample.



Deviation of signal from the base line.

* Applications in the separation of organic compounds :-

* In organic chemistry :-

① Separation of lipids :-

Lipids range from hydrocarbons and wax esters to highly polar sugar or phosphoric acid containing glyco and phospholipids. The polar head group interact with a polar stationary phase e.g, (silica) or non-polar chains interact with a non-polar stationary

phase to achieve separation of lipids by HPLC. Lipids yield distinct peaks from silica columns with elution gradient. Hydrocarbons elute first and then comes wax, cholesterol esters, glycerol ethers, triglycerides and free steroids etc.

② Steroids. Huber et al (1980) :-

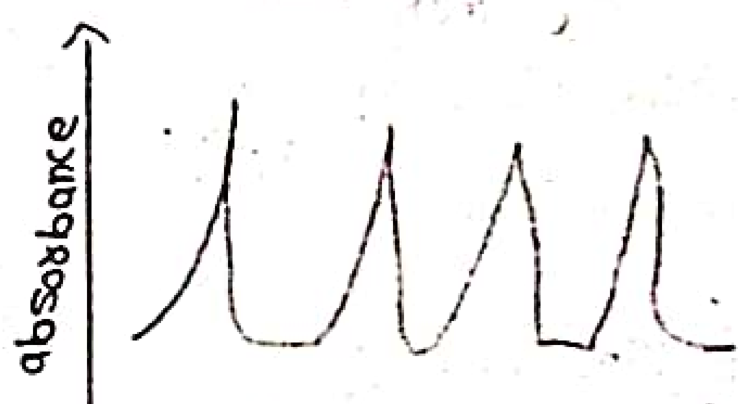
Huber et al (1980) have resolved mixtures of corticosteroids by liquid - liquid partition chromatography on zipax columns using mobile phase of heptane/tetrahydrofuran (80:20). Hesse and Hotzel (1974), Moffatt and Zordan (1977) have applied HPLC to determine corticosteroids in human plasma.

③ Achromatism of some water pollutents :-

Column :- 10 µm silica absorbed 100 x 4.6 mm

m.p :- CH_2Cl_2 , Flow rate :- 1 ml/min.

Detector :- UV - 253 nm

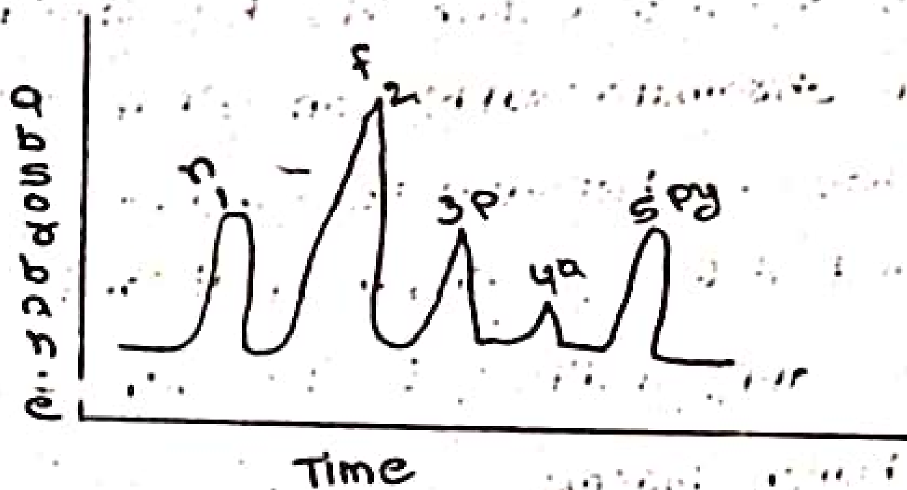


④ separation of polynucleus aromatic hydrocarbons :-

column :- 4.6 mm x 250 mm of totally porous spherical selica.

m.p :- acetonitrile / water (70 : 30)

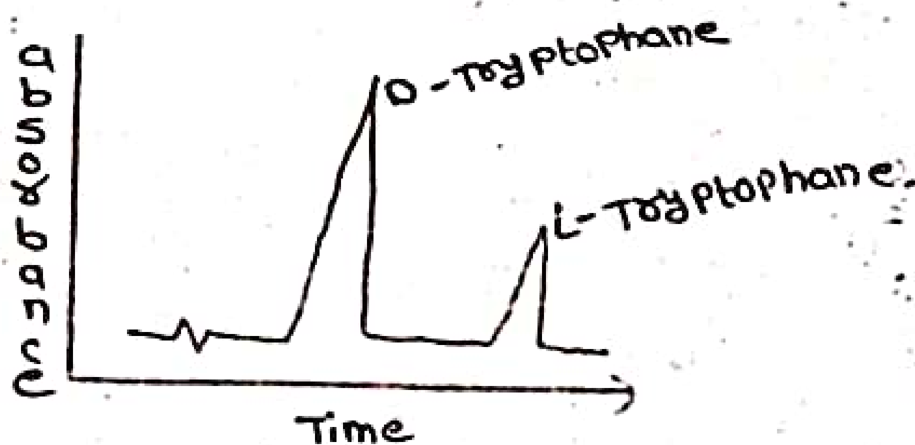
① naphtalene ② fluorene. ③. phenanthrene ④ anthrance ⑤ pyrene.



⑤ separation of D,L isomers of Tryptophan :-

column :- 25 cm x 0.46 cm ; flow rate :- 1.5 ml/min.

Temperature :- 50°c ; sample :- Tryptophane.



* LC - MS :-

mass spectrometer is the ideal detector for liquid chromatography because it is capable of providing both structural information and quantitative analysis for separated compounds. There are major problems in coupling of these two techniques.

* A gas phase sample is needed for mass spectrometry while the out put of the LC- column is a solute dissolved in a solvent.

* The solvent must be vapourised, when vapourise the LC- solvent produces a gas volume is 10-1000 times greater than its earlier volume.

* Another problum is the difficulty of vapourising in volatile and thermally labile molecules, without degrading them.

* The LC - system is maintained at high pressure conditions, where as the mass spectrometer needs vaccume conditions to be maintained.

So an interface is needed to connect the LC with ms. ✓ Sm